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DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

17811-014

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

10/031188

INTERNATIONAL APPLICATION NO.
PCT/IB00/01076INTERNATIONAL FILING DATE
20 July 2000 (20.07.2000)PRIORITY DATE CLAIMED
20 July 1999 (20.07.1999)

TITLE OF INVENTION

IMPROVED KERATINOCYTE CULTURE AND USES THEREOF

APPLICANT(S) FOR DO/EO/US

HUNZIKER, Thomas; LIMAT, Alain

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☒ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ has been communicated by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☐ is attached hereto.
 - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☒ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Express Mail Label No. EL918131156US
Filed On: 18 January 2002 (18.01.02)

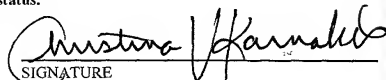
U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.101) 10/031188		INTERNATIONAL APPLICATION NO. PCT/IB00/01076		ATTORNEY'S DOCKET NUMBER 17811-014	
24. The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :				CALCULATIONS PTO USE ONLY	
<input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1040.00					
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Surcharge of \$130.00 for furnishing the oath or declaration later than _____ <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (c)).				\$0.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	51 - 20 =	31	x \$18.00	\$558.00	
Independent claims	- 3 =	2	x \$84.00	\$168.00	
Multiple Dependent Claims (check if applicable) <input type="checkbox"/>				\$0.00	
TOTAL OF ABOVE CALCULATIONS =				\$1,616.00	
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				\$808.00	
SUBTOTAL =				\$808.00	
Processing fee of \$130.00 for furnishing the English translation later than _____ <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (d)).				\$0.00	
TOTAL NATIONAL FEE =				\$808.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). <input type="checkbox"/>				\$0.00	
TOTAL FEES ENCLOSED =				\$808.00	
				Amount to be: refunded	\$
				charged	\$

- a. ☒ A check in the amount of **\$808.00** to cover the above fees is enclosed.
- b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **50-0311**. A duplicate copy of this sheet is enclosed.
- d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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45,899

REGISTRATION NUMBER

18 January 2002 (18.01.02)

DATE

IMPROVED KERATINOCYTE CULTURE AND USES THEREOF

FIELD OF THE INVENTION

The invention relates to the field of cell culture of human keratinocyte precursor and dermal fibroblast cells. The invention also relates to the use of cultured keratinocyte precursor cells in the repair of skin defects by skin grafting procedures.

BACKGROUND OF THE INVENTION

The healing of skin defects progresses through three general phases: (i) inflammation, (ii) wound cell migration and mitosis, and (iii) extracellular matrix production and remodeling. The ordered sequence of these events is thought to be orchestrated by interactions among cells, growth factors, and extracellular matrix proteins. A crucial step of skin wound healing is epidermal regeneration (*i.e.*, re-epithelialization). Besides interfollicular epidermal keratinocytes from the wound edges, the outer root sheath (ORS) cells from residual hair follicles also contribute to this process (*see e.g.*, Eisen *et al.*, 15 J. Invest. Dermatol. 145-155 (1955)). The ORS of hair follicles is comprised largely of undifferentiated keratinocytes that encompass the cylindrical structures of the hardened inner root sheath and the hair shaft (*see e.g.*, Montagna & Parakkal, In: *The Structure and Function of Skin* 172-258 (Academic Press New York, NY, 1974)). Recent literature has also indicated that ORS cells are at a lower level of commitment to differentiation than the basal interfollicular keratinocytes (*see e.g.*, Coulombe *et al.*, 109 J. Cell Biol. 2295-2312 (1989); Limat *et al.*, 194 Exp. Cell Res. 218-227 (1991); Limat *et al.*, 275 Cell Tissue Res. 169-176 (1994)), and label-retaining cells have been detected in the animal as well as the human ORS region near the bulge area which possibly represent stem cells for skin epithelial tissues (*see e.g.*, Cotsarelis *et al.*, 61 Cell 1329-1337 (1990); Kobayashi *et al.*, 90 Proc. Nat. Acad. Sci. USA 7391-7395 (1993); Yang *et al.*, 105 J. Invest. Dermatol. 14-21 (1993); Rochat *et al.*, 76 Cell 1073-1076 (1994); Moll, 105 J. Invest. Dermatol. 14-21 (1995)). Additionally, human ORS cells which are isolated from plucked anagen scalp hair follicles can be expanded extensively *in vitro* (*see e.g.*, Weterings *et al.*, 104 Brit. J. Dermatol. 1-5 (1981); Limat & Noser, 87 J. Invest. Dermatol. 485-488 (1986); Imcke *et al.*, 17 J. Am. Acad. Dermatol. 779-786 (1987);

Limat *et al.*, 92 J. Invest. Dermatol. 758-762 (1989)). Under conventional submerged culture conditions, ORS cells resemble interfollicular epidermal keratinocytes by both morphologic and biochemical (*e.g.*, keratin profiles) criteria (*see e.g.*, Stark *et al.*, 35 Differentiation 236-248 (1987); Limat *et al.*, 92 J. Invest. Dermatol. 758-762 (1989);
5 Limat *et al.*, 642 Ann. N.Y. Acad. Sci. 125-147 (1991)). In organotypic co-cultures with human dermal fibroblasts (*i.e.*, under conditions mimicking the epidermal environment), ORS cells with respect to histological, immunohistological, ultrastructural and biochemical criteria develop a stratified epithelium reminiscent of regenerating epidermis (*see e.g.*, Lenoir *et al.*, 130 Dev. Biol. 610-620 (1988); Limat
10 *et al.*, 194 Exp. Cell Res. 218-227 (1991); Limat *et al.*, 642 Ann. N.Y. Acad. Sci. 125-147 (1991)). If such organotypic cultures are grafted onto nude mice, ORS cells form a regular neo-epidermis that is under homeostatic control (*see e.g.*, Limat *et al.*, 59 Transplantation 1032-1038 (1995)). Thus, human ORS cells are of considerable interest for clinical application.

15 In the previous decade, interest has focused on the use of cultured epithelial cells for wound coverage. First, sheets of cultured autologous interfollicular keratinocytes were grafted successfully on acute wounds, mainly in the treatment of larger third degree burns (*see e.g.*, O'Connor *et al.*, 1 Lancet 75-78 (1981); Compton
20 *et al.*, 60 Lab. Invest. 600-612 (1989)) but also of epidermolysis bullosa (*see e.g.*, Carter *et al.*, 17 J. Am. Acad. Dermatol. 246-250 (1987)), pyoderma gangrenosum (*see e.g.*, Dean *et al.*, 26 Ann. Plast. Surg. 194-195 (1991); Limova & Mauro, 20 J. Dermatol. Surg. Oncol. 833-836 (1994)), and wounds after excision of giant congenital nevi (*see e.g.*, Gallico *et al.*, 84 J. Plast. Reconstr. Surg. 1-9 (1989)) or separation of conjoined twins (*see e.g.*, Higgins *et al.*, 87 J. Royal Soc. Med. 108-109
25 (1994)).

In contrast to the treatment of such acute wounds, the grafting of chronic wounds (*e.g.*, leg ulcers) with cultured keratinocytes has been much less successful. Allografts do not result in a permanent "take" (*see e.g.*, Fabre, 29 Immunol. Lett. 161-166 (1991)) and thus may be classified as a "quite effective but expensive biological
30 dressing" (*see Phillips et al.*, 21 J. Am. Acad. Dermatol. 191-199 (1989)). A reproducible, major definite "take" of autologous keratinocyte grafted by various modalities including: sheets of submerged keratinocyte cultures consisting of only a

few, noncornified cell layers (Hetton *et al.*, 14 J. Am. Acad. Dermatol. 399-405 (1986); Leigh & Purkis, 11 Clin. Exp. Dermatol. 650-652 (1986); Leigh *et al.*, 117 Brit. J. Dermatol. 591-597 (1987); Harris *et al.*, 18 Clin. Exp. Dermatol. 417-420 (1993)), trypsinized single cells attached to collagen-coated dressings (Brysk *et al.*, 25 J. Am. Acad. Dermatol. 238-244 (1991)), skin equivalents (Mol *et al.*, 24 J. Am. Acad. Dermatol. 77-82 (1991)) has yet to be convincingly documented within the scientific literature. The same lack of quantitative findings also holds true for various reports on the grafting of freshly isolated, autologous interfollicular keratinocytes (Hunyadi *et al.*, 14 J. Dermatol. Surg. Oncol. 75-78 (1988)) or ORS cells (Moll *et al.*, 46 Hautarzt 548-552 (1995)) fixed to the wound bed by the use of a fibrin glue. However, it should be noted that the disadvantages of the bovine serum used during cultivation of the keratinocytes may contribute to reduced "take" rate, due to the fact that it resists in keratinocytes (*see e.g.*, Johnson *et al.*, 11 J. Burn Care Rehab. 504-509 (1990)).

SUMMARY OF THE INVENTION

Prior to the disclosure of the present invention herein, the standard methodology for the generation of a primary culture of ORS keratinocytes consisted of the plucking of an anagen (*i.e.*, growing hair shaft) hair followed by a careful microscopic dissection to remove the hair bulbs and the infundibular hair shaft. The resulting outer root sheath was then placed on the culture insert for initiation of the primary keratinocyte culture. However, numerous subsequent studies (approximately 200), wherein the anagen hair was placed directly on the culture insert without performing the initial micro-dissection to remove the hair bulbs and the infundibular hair shaft, have demonstrated that such tedious and time-consuming dissection of the plucked anagen hair was not required. This has served to markedly simplify the handling process, reduce the risk for contamination, and resulted in more efficient initiation of keratinocyte cell plating.

Accordingly, it is an object of the present invention to provide improved and simplified methods for the generation of keratinocytes or keratinocyte precursors from outer root sheath cells (ORS cells) in fully defined culture conditions for the treatment of various types of skin defects (*e.g.*, chronic wounds such as leg ulcers, diabetic

- 3A -

DE-A-19651992 describes the culture of outer root sheath cells in 10-15% autologous or homologous serum to produce dermal equivalents. The dermal equivalents may be seeded on hyaluronic acid membranes or other biodegradable material prior to transplantation in order to optimise handling.

Lenoir-Viale, M. C. (Arch. Dermatol. Res. 1993, 285: pages 197-204) describes the *in vitro* preparation of a reconstructed epidermis from the outer root sheath of human hair follicles. The reconstructed epidermis is described as a valuable and promising tool for pharmacological studies and may represent a model of wound-healing.

Limat, A. (J. of Investigative Dermatology 2000, Nov. 7, pages 128-134) describes the culturing of hair follicles (hair bulbs and infundibular parts removed) to generate epidermal equivalents and the use thereof for treating chronic leg ulcers.

SUMMARY OF THE INVENTION

Prior to the disclosure of the present invention herein, the standard methodology for the generation of a primary culture of ORS keratinocytes consisted of the plucking of an anagen (i.e., growing hair shaft) hair followed by a careful microscopic dissection to remove the hair bulbs and the infundibular hair shaft. The resulting outer root sheath was then placed on the culture insert for initiation of the primary keratinocyte culture. However, numerous subsequent studies (approximately 200), wherein the anagen hair was placed directly on the culture insert without performing the initial micro-dissection to remove the hair bulbs and the infundibular hair shaft, have demonstrated that such tedious and time-consuming dissection of the plucked anagen hair was not required. This has served to markedly simplify the handling process, reduce the risk for contamination, and resulted in more efficient initiation of keratinocyte cell plating.

Accordingly, it is an object of the present invention to provide improved an simplified methods for the generation of keratinocytes or keratinocyte precursors from outer root sheath cells (ORS cells) in fully defined culture conditions for the treatment of various types of skin defects (e.g., chronic wounds such as leg ulcers, diabetic

ulcers, pressure sores, and the like) in both humans and animals. In addition to their use in the treatment of wounds, keratinocytes may also be used in plastic and cosmetic surgery, or whenever there is a demand for such skin support (e.g., post operative following the removal of tattoos, naevi, skin cancer, papillomas, after
5 amputation, in sex transformation or re-virgination, rejuvenation of actinically damaged skin after skin resurfacing, tympanoplasty, epithelialization of external ear canal, and the like).

These aforementioned objectives are accomplished by explantation and culture of plucked, anagen or growing hairs *in toto* upon microporous membranes carrying human fibroblast feeder cells at their under-surface. In such primary cultures, large
10 numbers of ORS cells can be easily and repeatedly obtained, irrespective of the donor's chronological age. Such ORS cells may be used for the subsequent preparation of complex skin, *i.e.*, dermo-epidermal, or epidermal equivalents or kept frozen and stored in order to use them at a later time point.

The subsequent preparation of skin or epidermal equivalents is achieved by the "seeding" of these ORS cells upon a modified, microporous membrane carrying fibroblast feeder cells (most preferably growth-arrested/limited human dermal fibroblast "feeder cells") at their under-surface. During culture, these ORS cells
15 undergo tissue differentiation which has been demonstrated to be similar to that of normal epidermis. This finding is most probably due to a large compartment of proliferating cells. The modified culture conditions which are disclosed herein are important for the successful treatment of chronic wounds with epidermal equivalents generated *in vitro* from autologous ORS cells.
20

A further object of the present invention is to provide improved culture
25 systems for ORS-derived keratinocytes by adhering the anagenic hair onto a polymeric microporous membrane coated with one or more molecules of extracellular matrix origin. These improved cultures of ORS cells, designated as skin equivalents or epidermal equivalents, may be used to treat skin defects, especially chronic wounds.

Yet another object of the present invention is to produce skin or epidermal
30 equivalents using a reduced concentration of allogenic or homologous serum. This greatly mitigates the risk of disease transmission, for example, by clinical use of

blood products, by the use of autologous or homologous human serum and substances derived or released from blood components (*e.g.*, blood platelets) for supplements in *in vitro* culturing steps.

5 A further object of the present invention is a methodology which reduces the probability of mechanical damage (*e.g.*, separation of the various constituent layers) of the skin or epidermal equivalents during transport prior to transplantation.

10 The clinical advantages of the methodology of the present invention, as compared to grafting techniques of chronic wounds which have been previously utilized, include, but are not limited to: noninvasiveness (so that the cells are available repeatedly), the lack of need for surgical facilities or anesthesia during the grafting procedure, and a short immobilization period of only 2 hours required following the grafting procedure.

DETAILED DESCRIPTION OF THE INVENTION

15 Unless defined otherwise, all technical and scientific terms used herein have the same meanings commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, the preferred methods and materials are now described. All publications mentioned herein
20 are incorporated herein in their entirety by reference.

The term "keratinocyte layer" as used herein means an *in vitro* generated keratinocyte tissue culture with more or less differentiated structure. The term "epidermal equivalent" as used herein means an *in vitro* generated organotypic tissue culture resembling in its histological structure the natural epidermis especially
25 concerning the stratification and development of the horny layer. A normal stratified epidermis consists of a basal layer of small cuboidal cells, several spinous layers of progressively flattened cells, a prominent granular layer and an orthokeratotic horny layer. All these layers can be detected in the epidermal equivalents that are subject of the invention. Localization of those epidermal differentiation products that have been
30 assayed by immunohistochemistry (*e.g.* keratins, involucrin, filaggrin, integrins) is similar to that found in normal epidermis.

The term "autologous" as used herein means: (i) that biological material to be transplanted is derived from the individual to be treated with epidermal equivalents; or (ii) that biological material added to tissue cultures comes from the donor of the cells for tissue culture.

5 The term "homologous" as used herein means: (i) that biological material to be transplanted is derived from one or more individuals of the same species as the individual to be treated with epidermal equivalents; or (ii) that biological material added to tissue cultures comes from one or more individuals of the same species as the donor of cells for the tissue culture.

10 The term "organotypic culture" and the like, refers to culture of cells under conditions that promote differentiation of the cells. Under conditions of organotypic culture, proliferation of the cells is slowed compared to culture under "proliferative" conditions such as primary culture conditions, and may be completely stopped. In the present case, an important condition for organotypic culture is maintenance of the cells at the air-liquid interface, a so-called "lifted" culturing condition.

15 The term "releasate from blood components" (e.g., blood platelets) as used herein means any combination of cytokines or other growth factors obtained from blood components (e.g., blood platelets). Platelets stimulated with, for example, thrombin release the content of their alpha granules into the surrounding medium.

20 Alpha granules usually contain several cytokines (e.g., platelet derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factors alpha and beta (TGF alpha/beta), platelet factor 4 (PF-4), platelet basic protein (PBP)). However, it is possible to obtain cytokines and other growth factors from platelets by other methods than stimulating with thrombin. Moreover, other blood components produce growth factors and cytokines as well. Monocytes, for example, produce IL-1, TNF alpha, IL-25 6 and other substances of interest.

General Method for Preparing Epidermal Equivalents from ORS Cells.

30 Keratinocyte precursor cells are selected from outer root sheath (ORS) of anagen or growing hair which is derived from the individual which is to be subsequently treated with epidermal equivalents. In general, approximately 40 hair follicles are plucked from the scalp. and those in the anagen phase (i.e., a growing hair shaft) are then selected under the dissecting microscope. A total of four weeks of culture is usually

required in order to obtain approximately 1 cm^2 of epidermal equivalents from five hair follicles. However, with improved culture and fermentation techniques it may be possible to get a higher yield (*i.e.*, a larger area of epidermal equivalents, within this period of time).

5 The previous standard method for the generation of a primary culture of ORS keratinocytes consisted of the plucking of an anagen (*i.e.*, growing hair shaft) hair followed by a careful microscopic dissection to remove the hair bulbs and the infundibular hair shaft. The resulting outer root sheath (ORS) was then placed on the culture insert for initiation of the primary keratinocyte culture. However, numerous
10 subsequent studies (approximately 200), wherein the anagen hair was placed directly on the culture insert without performing the initial micro-dissection to remove the hair bulbs and the infundibular hair shaft, have demonstrated that such tedious and time-consuming dissection of the plucked anagen hair was not required. This has served to markedly simplify the handling process, reduce the risk for contamination, and
15 resulted in more efficient initiation of keratinocyte cell plating.

 The selected anagen hairs were incubated in an appropriate rinsing buffer containing various anti-microbial and anti-fungal agents (*e.g.*, fungizone, penicillin, and streptomycin). Following this procedure, the entire plucked anagen hair is placed
20 directly on the culture insert and allowed to grow for several days, preferably 7-14, days, and more preferably 8 to 10 days. An optional, additional step is comprised of passaging the primary culture and performing a secondary culture in order to obtain more cellular material for the preparation of larger areas of epidermal equivalents.

 The culture insert, a microporous membrane coated with one or more extracellular matrix substances (*e.g.*, fibrin, fibronectin, collagens, laminins or
25 hyaluronan or mixtures thereof), carries a growth-arrested/limited feeder cell system on its undersurface. The coating of the membrane insert with such extracellular matrix substances provides for: (i) an enhanced culture surface for the initial attachment of the anagen hair (*i.e.*, it sticks easily and remains stationary); (ii) a surface which significantly enhances the migration of the ORS keratinocytes away from the outer
30 root sheath (ORS) anagen hair follicles; and (iii) increased growth rates of the spreading ORS keratinocytes (*i.e.*, the overall culture time needed for production of

fully differentiated skin or epidermal equivalents) can be reduced to three weeks, instead of four.

The aforementioned growth-arrested/limited feeder cell system located on the under surface of the microporous insert membrane is comprised of primary dermal fibroblasts obtained from a human skin biopsy. The primary dermal fibroblasts are treated with mitomycin-C for 4 to 6 hours prior to their use as a "feeder cell layer" for the plucked anagen hair and then plated on the underside of the culture insert. Growth arrest/limitation is induced by either mitomycin-C or X-ray treatment or, preferably, the reduced serum concentration below 5%, and preferably 2%. It should be noted that, although some cultures had been performed using 10% fetal calf serum (FCS; Boehringer Mannheim, Germany), the current utilization of human serum, in order to reduce the number of allogeneic ingredients, was found to provide markedly superior outgrowth and proliferation of the ORS cells. Moreover, the human serum is preferably utilized in a concentration of less than 5%, and more preferably in a concentration of 2%. In the presence of such low serum concentrations, the primary human dermal fibroblasts of the present invention will become significantly, or completely growth arrested. Hence, in this manner, two expensive and potentially complicating steps in the autologous ORS culture system may be removed. The two complicating steps include: (i) removal of high serum >5% concentrations, which reduces the overall cost of the process significantly and; (ii) the removal of mitomycin-C treatment, which provides a fully mitomycin-C-free culture system and eliminates any concerns regarding the total elimination of the drug from the primary culture inserts prior to the growth of the epidermal equivalents. In addition, the use of reduced serum concentrations allows the alternative feeder cell-arresting procedure (i.e., the X-ray exposure step) to be eliminated, thus saving significant time and expense in the overall procedure.

Following expansion of the ORS cells to an appropriate density (i.e., 1×10^3 to 1×10^6 cells/cm², and preferably 5×10^4 to 1×10^5 cells/cm²), they are used for preparation of epidermal equivalents. Preferably, the cells are grown to confluence. The epidermal equivalents are prepared by seeding ORS cells at an appropriate cell density (i.e., 30×10^3 to 100×10^3 cells/cm², and preferably 60×10^3 cells/cm²) within a culture device which is suitable for "lifting" the cells up to the air-liquid interface

during culture. Subsequently, one to four days after seeding (preferably 3 days after seeding), the ORS cells are exposed to air (e.g., by aspiration of the medium inside the insert) and the cultures are then continued for approximately 10-20 days, and preferably for 14-18 days, in such "lifted" culture condition. The medium is changed periodically during the lifted culture; preferably every two to three days.

The present invention also encompasses skin equivalents which include additional layers, and so are more complex structures than epidermal equivalents. Skin equivalents comprise differentiated ORS cells as their epidermal part and also a layer comprising a matrix component, preferably one containing embedded dermal fibroblasts and/or other cells (i.e., an "embedding matrix"). Skin equivalents are made by placing a matrix with one or more extracellular matrix substances (e.g., fibrin, fibronectin, collagens, laminins or hyaluronan or mixtures thereof) on the upper surface of the microporous membrane described above. When embedding human dermal fibroblasts, preferably autologous human dermal fibroblasts, the cells are embedded at a density of 1×10^3 to 1×10^7 cells/cm³; preferably 1×10^4 to 1×10^5 cells/cm³; and most preferably approximately 5×10^4 cells/cm³. The primary culture of ORS cells is then seeded on top of the matrix (preferably containing embedded dermal fibroblasts and/or other cells) and organotypic culturing is performed as described above. For a detailed description of the preparation of dermal equivalents (see e.g., Limat *et al.*, 194 Exp. Cell Res. 218-277 (1991)).

It should be noted, however, that the cells which are embedded in the matrix need not be limited exclusively to dermal fibroblasts; as epidermal, mesenchymal, neuronal and/or endothelial cells can also be utilized. The embedded cells are preferably obtained from skin tissue, are more preferably allogeneic cells, and are most preferably autologous cells.

All culture steps are performed in an appropriate medium which allows the proliferation of the ORS cells and their outgrowth from the hair follicles, the medium is typically changed every 2 to 3 days. Generally, the medium utilized for all steps is the same. The medium is typically based on a minimal medium and contains several additional ingredients. One common ingredient is serum in a concentration of 0.5-60%. In the preferred embodiment of the present invention, human serum is used at a concentration of less than 5%, and most preferably at a concentration of 2%.

Furthermore, with the development of serum-free media, it may be possible to omit serum *in toto*. Epidermal growth factor (EGF) stimulates migration of keratinocytes and delays their senescence which results in stimulation of proliferation. Cholera toxin, hydrocortisone, insulin, adenine and triiodothyronine have an effect of stimulating proliferation. All of these ingredients are thus useful in a medium for preparing epidermal equivalents. Nevertheless, it may be possible to omit or replace one or another of these ingredients.

Releasate from blood components (*e.g.*, blood platelets, monocytes or lymphocytes), may serve as a source of cell proliferating activities, and therefore may substitute serum and provide other above mentioned ingredients. For certain culture periods the serum-containing medium might possibly be replaced by a defined, serum-free medium, for example, SFM (Gibco Europe, Ettlingen). The releasate from blood components (*e.g.*, blood platelets, monocytes or lymphocytes), especially of homologous or autologous origin, may serve as a source of cell proliferating activities and therefore may substitute serum and provide other above mentioned ingredients or indeed may provide additional ingredients. The blood components should be added to the culture medium in a concentration of 0.1% to 20%, and preferably 1% to 5%, after the releasate is brought to the same final volume as the blood from which these components are obtained. These releasates contain several growth factors that are present in serum (*e.g.*, PDGF, ECF or TGFs). However, serum as well as releasates contain many substances, and not all are characterized.

Releasate from blood platelets is obtained by centrifugation of anti-coagulated whole blood, preferably human blood, in order to pellet all cells except thrombocytes. The supernatant is centrifuged once more to spin down the thrombocytes. The thrombocytes are suspended in an appropriate buffer, *e.g.* phosphate buffer and treated with thrombin in order to release their alpha granules which contain a mixture of various growth factors (*e.g.*, PDGF, PF-4, TGF- β , EGE, β -thromboglobulin). In a further centrifugation step all cellular material is removed. Finally, the supernatant is supplemented with buffer to the volume of the original blood sample from which the components are obtained. The blood components should be added to the culture

medium in a concentration of 0.1% to 20%; preferably 1% to 10%; and more preferably 2 to 5%.

Similarly, releasates can be obtained from other blood cells, such as monocytes, by breaking up the cells (*e.g.*, by sonication, freeze-thaw method, or the
5 like) and purifying the growth factors (*e.g.*, by filtration or immunological methods).

The blood component releasates can also be used to condition the wound bed in the course of grafting the epidermal or dermal equivalents. Furthermore, the culture medium containing the releasates and used to perform the organotypic culturing step, after having been conditioned by the cells, can be used to condition the bed of the skin defect in the course of grafting the epidermal or dermal equivalents.
10

Cultivation usually is performed in inserts with microporous membranes, which contain homologous or autologous human dermal fibroblasts (HDF), especially postmitotic HDF at their undersurface. HDF secrete factors that condition the medium in order to get a better growth of the epidermal equivalents. The HDF layer can be
15 formed from between 5×10^3 to 1×10^5 cells/cm², and preferably approximately 1×10^4 to 5×10^4 cells/cm². The HDF are preferably postmitotic, but earlier passage cells can be used if they are irradiated, treated with mitomycin-C, or otherwise treated to inhibit their proliferation but maintain their metabolism, *i.e.*, by reduction of serum concentration.

20 In one embodiment, the graft thickness for the complex dermal ("complex skin") equivalents does not exceed 0.4 mm.

Microporous membranes are suitable as a culture substrate, because they allow substances to diffuse from one side to the other, but work as a barrier for cells. The pore size of the membrane is not a limitation on the present invention, but should be
25 adequate so as to allow diffusion of proteins of up to 100,000 Daltons molecular weight, and preferably of up to 70,000 Daltons molecular weight. The membrane should at least allow diffusion of small hormones such as insulin, and allow passage of proteins of up to 15,000 Daltons molecular weight. Other means than a microporous membrane for performing the function of allowing diffusion of soluble
30 factors to the cultured ORS cells, while preventing mixing of the ORS cells with the HDF would also be usable.

The microporous membranes typical in the art are usually used. However, membranes fabricated from a biodegradable material (*e.g.*, polyhyaluronic acid or polylactic acid) can also be used. When a biodegradable microporous membrane is employed it is contemplated that the entire culture, including the differentiated ORS cells, the microporous membrane and the HDF, will be transplanted into the skin defect. Thus, in this alternative embodiment, the HDF grown on the underside of the membrane need not be post-mitotic or treated to preclude proliferation. While HDF tend to be less immunogenic than keratinocytes, it is preferable that when this embodiment is employed, the HDF be allogeneic cells, preferably autologous cells.

In one embodiment, the thickness of mesh graft can range from 30-300 microns. Preferably, the mesh graft thickness ranges from 0.5-0.75 mm. A graft of tissue (for example, dermal collagen plus fibroblasts overlaid with keratinocytes tissue) that is too thick can result in a too rapid ischemic cell death, especially for the keratinocyte layer residing above the dermal fibroblast collagen layer. By contrast, this mesh graft tissue can "take" in wound sites.

The epidermal equivalents of the present invention may range in size from approximately 6 mm to approximately 2.5 cm in diameter, with a preferred diameter of 2.5 cm. For practical reasons, the experiments disclosed herein were performed with epidermal equivalents of approximately 2.5 cm in diameter.

In one embodiment, the preferred range for epidermal equivalents is 50-150 microns. In a particular embodiment, the epidermal equivalents are very thin (thinner than is generally used in the art, for example, 60 microns). It has been hypothesized that making the autologous graft too thick will prevent a proper blood supply from being established, so that the epidermis will not "take" at the wound site. By contrast, the epidermal equivalents of the invention can "take" in wound sites.

In many cases, however, the skin or epidermal equivalents will have to be delivered from the facility where they are generated to the institution where they are used. Therefore a system is needed to enable the transport of the skin or epidermal equivalents, which have been kept in a condition ready for grafting. Irrespective of whether the microporous membrane is removed from the basal cell layer before transport, conditions resembling those during cultivation seem to be favorable. In order to keep the skin or epidermal equivalents in contact with medium only from the

basal layer, (*i.e.*, during cultivation), agarose in a concentration ranging from 0.1% to 5%, and preferably in a concentration of 0.5% to 1%, or methyl cellulose, or any other gelifying substance in comparable concentrations, may be used to solidify the transport medium. The skin or epidermal equivalents will be placed with their basal layer down on the membrane of an insert previously embedded on top of the solidified or gelled medium. The multiwell dish containing these inserts is then put in a blister sealed by a tyvek cover, and shipped. The skin or epidermal equivalents are, most preferably, used for grafting within 24 to 48 hours of initial packaging.

To improve the stability of the epidermal equivalents, the technique of placing a carrier membrane on top, *i.e.*, onto the cornified aspect, of the epidermal equivalents and eventually adhering to it was developed. As an adhesive, fibrin glue is preferred, however, other options, including, but not limited to: extracellular matrix components such as collagen, fibronectin, proteoglycans (*e.g.*, hyaluronic acid, chondroitin sulfate, and the like), or basement membrane zone components (*e.g.*, laminin, Matrigel™, or L-polylysine), or similar tissue glues, may also be utilized.

The carriers utilized in the present invention may consist of a synthetic membrane, made from at one or more of the following materials (polyester, PTFE or polyurethane); from one or more biodegradable polymers (*e.g.*, hyaluronic acid, polylactic acid or collagen); or a silicone or vaseline gauze dressing, or any other material suitable for wound dressing. These materials which are suitable for wound dressing allow the carrier to remain in place to immobilize the implanted dermal or epidermal equivalents for several days, rather than requiring the carrier to be removed immediately after the dermal or epidermal equivalents are transplanted. Thus, the carrier not only enhances stability and improves handling, but it also serves as a protective coat against physical damage as well as the proteolytic milieu and bacteria in the wound. Moreover, it serves for orientation of the graft (*i.e.*, basal side down, cornified side up).

The skin or epidermal equivalents put onto the carrier have to be kept in a condition ready for grafting. Irrespective of whether the microporous membrane is removed from the basal cell layer for transport, conditions resembling those during cultivation seem to be favorable. In order to keep the skin or epidermal equivalents in contact with medium only from the basal layer (*i.e.*, during cultivation), agarose in a

concentration ranging from 1% to 5%, and preferably in a concentration of 1 to 3%; methyl cellulose; or any other gelifying substance in comparable concentrations, may be used to solidify the medium. The epidermal equivalents together with the carrier will be placed with their basal layer on top of the solidified or gelled medium. The whole device is then sealed in an air tight manner, and shipped. The epidermal equivalents are, most preferably, used for grafting within 24 hours of initial packaging.

The skin or epidermal equivalents are transplanted by simply placing them in the bed of the wound or other skin defect. Preferably the skin or epidermal equivalents are then immobilized (patients are immobilized for 2 hours). The preferred method for immobilization is by use of a biodegradable material, by some sort of tissue glue or adequate bandage. As previously described, the bed of the skin defect can be treated with blood releasates or the medium from the organotypic culturing prior to, or concomitantly with, the transplantation.

In work using encapsulated cells devices (100 micron membrane, 200-250 microns to the center of the hollow fiber), good survival of human dermal fibroblasts has been obtained at 300 micron distances from the nearest blood vessel.

EXAMPLE 1 PREPARATION OF ORS CELLS

Keratinocyte precursor cells from the outer root sheath (ORS) of the hair follicles are selected and subsequently cultured by use of the following methodology, as disclosed in the present invention.

Approximately 40 hair follicles were plucked with tweezers from the occipital scalp of individuals, and those in the anagen phase, as detected, for example, by well-developed root sheaths, were then selected under the dissecting microscope (*see e.g.*, Limat & Noser, 87 J. Invest. Dermatol. 485-488 (1986); Limat *et al.*, 92 J. Invest. Dermatol. 758-762 (1989)). The anagen hair was placed directly on the microporous culture insert without performing the previously-utilized micro-dissection to remove the hair bulbs and the infundibular hair shaft.

Generally, six anagenic hairs were explanted on the microporous membrane of a cell culture insert (Costar) that carried on its undersurface a preformed feeder layer

preferably comprised of 20×10^3 postmitotic human dermal fibroblasts (HDF) per cm^2 . (see e.g., Limat *et al.*, 92 J. Invest. Dermatol. 758-762 (1989)). The HDFs were derived from skin explants of a healthy, repeatedly HIV- serology negative and hepatitis-serology negative individuals and cultured in DMEM supplemented with
5 10% fetal calf serum (FCS), or preferably less than 5% human serum, or most preferably 2% human serum.

For the purpose of obtaining an efficient outgrowth of the outer root sheath (ORS) cells from the anagen hair and a high proliferation rate, it is important not to place the HDF feeder cells at the bottom of the culture dish, resulting in an additional
10 medium layer between the HDF layer and the microporous membrane supporting the ORS cells. Growing each cell type at one side of the microporous membrane allows a very close interaction, but prevents cross contamination of the ORS cells with fibroblasts and thus guarantees a pure culture of ORS cells.

The culture medium which was utilized consisted of Dulbecco's modified Eagle's medium/F12 (3:1 v/v) supplemented with 2% human serum, 10 ng of
15 epidermal growth factor per ml of culture medium, 0.4 microgram of hydrocortisone per ml, 0.135 mM adenine, and 2 nM triiodothyronine (all obtained from Sigma Chemical Co., St. Louis, MO). The preferred final Ca^{2+} concentration of the culture medium is 1.5 mM (see e.g., Wu *et al.*, 31 Cell 693-703 (1982); Limat & Noser, 87 J.
20 Invest Dermatol. 485-488 (1986)). Within about 2 weeks, the ORS cells had expanded and reached confluence. They were dissociated with 0.1% trypsin/0.02% EDTA mixture, checked for viability, and used for preparation of epidermal equivalents. It should be noted that, although initial cultures had been performed using 10% fetal calf serum (FCS; Boehringer Mannheim, Germany), current utilization of human serum.
25 in order to reduce the number of allogeneic ingredients, provided superior outgrowth and proliferation of the ORS cells. The human serum is preferably utilized in a concentration of less than 5%, and most preferably in a concentration of 2%.

Explanting plucked anagen hairs directly on the membrane of culture inserts carrying postmitotic HDF on the undersurface as feeder cells proved to be a simple.
30 efficient, and reproducible method for establishing primary cultures of ORS cells. Approximately 80% of the explanted hair follicles gave rise to outgrowth of ORS cells, even when derived from individuals aged more than 90 years. After 14 days.

large areas of the insert were covered by compactly arranged small cells, at which time they were used for preparation of epidermal equivalents of the present invention.

The comparison of the growth behavior of 70 strains of ORS cells, which were derived from a total of 30 donors, demonstrated no significant differences between the young (*i.e.*, 21 donors aged 19-50 years) and the old (*i.e.*, 9 donors aged 51-93 years) donors. Approximately 5×10^5 cells were generally obtained per explanted follicle and the overall degree of cell viability was typically higher than 95%. In contrast, in the absence of postmitotic HDF as a feeder layer, there was only sporadic outgrowth of ORS cells from the explanted follicles.

EXAMPLE 2 PREPARATION OF EPIDERMAL EQUIVALENTS

ORS cells harvested from primary cultures were seeded at a density of 30×10^3 cells/cm² to 100×10^3 cells/cm², and preferably 60×10^3 cells/cm², on cell culture inserts (Costar) which had been previously inoculated with 10×10^3 cells/cm² to 50×10^3 cells/cm², and preferably 20×10^3 cells/cm², of postmitotic HDF cells on the undersurface of their microporous membrane. Similar to the culture of ORS cells, it is important to keep the HDF feeder cells in close proximity with the ORS cells, while concomitantly keeping them separated by use of the microporous membrane. This culture technique enhances proliferation, differentiation, and thus the homeostasis of the developing tissue.

Culture medium was identical that that utilized for the preparation of the primary cultures as described *supra*. After 72 hours, the ORS cells were exposed to air by aspiration of the liquid medium inside the insert (*i.e.*, leaving the underside of the insert in contact with medium) and cultured for an additional 12-14 days, with three medium changes per week. Alternatively, after one week lifted culture serum may be totally omitted.

For transplantation, the so-far-utilized protocol, which is generally employed for preparation of the fully differentiated epidermal equivalent for wound grafting, requires the physician to carefully cut the entire perimeter of the culture insert with a scalpel blade so as to facilitate the release of the insert membrane (with undercoated human dermal fibroblasts) with the attached skin patch squamous-side up. The skin

patch is then released from this membrane by peeling with a fine forceps and placed, basal-side up, on a new membrane disk in a culture dish for eventual transplant to the patient. This aforementioned procedure is both laborious and time consuming, and can lead to reversal of the basal and squamous orientation.

5 A markedly simpler method which utilizes a carrier membrane patch cap has been devised which utilizes a membrane patch cap (analogous to the fibrin glue patch procedure described below) which is placed directly on top of the squamous surface layer. The membrane cap can then be easily grasped together with the skin patch below using fine forceps and peeled from the culture insert well surface, and, *e.g.*,
10 after incubation in diluted Dispase solution, be peeled from the culture insert membrane. The membrane can then serve a plate for placing the graft onto the wound without mixing up the orientation of the graft (*i.e.*, basal side down, squamous side up).

For stabilization and as a protective coating in case of grafting, the epidermal
15 equivalents of the present invention are coated on top with diluted fibrin glue, which also serves to clearly identify the upper (*i.e.*, cornified) side. Fibrin glue, the preferred embodiment of the present invention, is a generally accepted, natural human product which is used extensively as a tissue glue. By applying a thin coating of fibrin glue (which is clearly visible with the naked eye) to the cornified squamous air-exposed
20 surface of the epidermal equivalent, the physician placing the epidermal equivalent onto the wound site will be fully assured of proper graft orientation (*i.e.*, the basal surface of the skin patch will always be the side that does not have the clearly visible fibrin glue cap). Previously, in many instances, during the preparation of the epidermal patch for wound grafting, the orientation of the patch becomes confused.
25 Should the skin patch be placed in squamous-side down orientation onto the graft site, there would be significantly decreased likelihood of a successful graft. Thus, the use of this simple "marking" completely eliminates this problem.

In addition, anti-microbial and/or anti-fungal substances may also be included
30 in the fibrin glue, so as to impede any possible microbial contamination or overgrowth of the graft. Many chronic lesions are chronically-infected, which can result in the inhibition of graft "take" and subsequent wound healing following the initial skin grafting. Moreover, the addition of one or more antibiotics or anti-fungal agents by

direct emulsification within the fibrin glue surface cap, may provide a significant improvement in the delivery of sufficient quantities of anti-microbial agents to the transplant site.

It should be noted that the ORS cells which were harvested from primary cultures, and cultured at the air-liquid interface on insert membranes carrying postmitotic HDF at their undersurface, typically developed a stratified epithelium within 14 days. This stratified epithelium consisted of a basal layer of small cuboidal cells below a thick suprabasal compartment of progressively flattened cells. A prominent granular layer, as well as an orthokeratotic horny layer were also found to be present.

Based upon the experimental finding of approximately 80% of the follicles giving rise to ORS cell outgrowth, approximately five anagen hair follicles were required for the generation of 1 cm² of epidermal equivalents. The period to generate graftable epidermal equivalents usually was four weeks *in toto* (i.e., two weeks for the primary culture and two weeks for the subsequent organotypic culture).

EXAMPLE 3 STABILIZATION

Before delivery, the epidermal equivalents are "coated on-top" by placing a silicone membrane of an appropriate diameter onto the cornified upper aspect of the cultures. To further enhance stability, e.g., in case of thin and/or large epidermal equivalents, as well as to increase adhesion of the silicone membrane, a thin layer of tissue glue, e.g. fibrin glue, may be applied before.

On-top coating (1) enhances stability and improves handling of the grafts, and (2) serves as a protective coat against physical damage as well as the proteolytic milieu and bacteria in the wound.

EXAMPLE 4 SHIPPING

On-top coated epidermal equivalents are detached from the culture insert membranes by incubation in diluted Dispase and then grasping the epidermal equivalents together with the silicone membrane using fine tweezers and transferring

them on the membrane of an insert previously embedded in 0.7% agarose soaked with culture medium in the well of a multiwell dish. These dishes are then placed in the shipping container. For application to the wound bed, the epidermal equivalents are again grasped, together with the silicone membrane, which (1) serves for orientation of the graft (*i.e.*, basal side down, cornified side up) and (2) by leaving it on the grafted epidermal equivalents in the wound serves as a protective coat (*see* above).

EXAMPLE 5
SUCCESSFUL TREATMENT OF CHRONIC LEG ULCERS WITH EPIDERMAL
EQUIVALENTS GENERATED FROM CULTURED AUTOLOGOUS OUTER
ROOT SHEATH CELLS

The outer root sheath cells of hair follicles can substitute for interfollicular epidermal keratinocytes, as during healing of skin wounds when these cells migrate onto the denuded area and contribute to epidermal regeneration (Limat *et al*, 107(1) J. Invest. Dermatol. 128-35 (1996), incorporated by reference). Using the improved culture techniques of the invention, we generated epidermal equivalents from cultured outer root sheath cells of patients suffering from recalcitrant chronic leg ulcers, primarily of vascular origin. In such epidermal equivalents, tissue organization as well as immunolocalization of epidermal differentiation products (keratin 10, involucrin, filaggrin) and integrins were indistinguishable from normal epidermis. As determined by the number of bromodeoxyuridine-incorporating cells, the basal layer contained a large compartment of proliferative cells irrespective of donor age. FACS analysis of the outer root sheath cells, used to prepare the epidermal equivalents, disclosed a fraction of small cells with enhanced expression of $\beta 1$ -integrin, a potential stem cell marker. In contrast to acute wounds, a major definitive take of grafted cultured autologous keratinocytes has not been convincingly demonstrated in chronic wounds. Grafting of epidermal equivalents generated *in vitro* from autologous outer root sheath cells on 11 ulcers in five patients resulted in a definitive take rate of about 80%, with subsequent complete healing within 2 to 3 weeks of five out of seven ulcers grafted with densely arranged cultures. This improvement in the treatment of chronic leg ulcers with cultured autologous keratinocytes probably depends on the large compartment of proliferative cells as well as on a well-developed horny layer

which prevents disintegration of the grafts. Practical advantages of the new technique are its noninvasiveness, the lack of need for surgical facilities or anesthesia, and a short immobilization period after grafting.

In Vitro Experiments. Cell Cultures. About 40 hair follicles were plucked from the occipital scalp of individuals aged up to 91 years, and those in the anagen phase selected under the dissecting microscope. The hair bulbs as well as the infundibular parts were removed with microsurgical blades. Usually, six follicles were explanted on the microporous membrane of a cell culture insert (Falcon 3090; Becton Dickinson, Franklin Lanes, NJ) that carried on its undersurface a performed feeder layer made of 10^5 postmitotic human dermal fibroblasts. Culture medium consisted of Dulbecco's modified Eagle's medium/F12 (3:1) supplemented with 10% fetal calf serum (Boehringer Mannheim, German), 10 ng of epidermal growth factor per ml, 0.4 μ g of hydrocortisone per ml, 0.1 nM cholera toxin, 0.135 mM adenine, and 2 nM triiodothyronine (all from Sigma Chemical Co., St. Louis, MO), final Ca^{2+} concentration 1.5 mM. Within about 2 wk, the ORS cells expanded and reached confluence. They were dissociated with trypsin/EDTA 0.1%/0.02%, checked for viability, and grown either in secondary cultures in keratinocyte growth medium (KGM containing 0.15 mM Ca^{2+} ; PromoCell, Heidelberg, Germany) or used for flow cytometry analysis and preparation of epidermal equivalents (*see, below*). For long-term storage in liquid nitrogen, they were frozen in KGM containing 10% fetal calf serum and 10% dimethylsulfoxide.

For comparison, primary cultures of ORS cells were also established by trypsinization of hair follicles and plating the disaggregated ORS cells on a preformed feeder layer made of postmitotic fibroblasts, as previously described (Limat *et al*, 1989). To avoid confusion, follicles obtained by this method are referred to as "trypsin-treated follicles."

Fibroblasts were derived from skin explants of a healthy, HIV-serology, and hepatitis-serology-negative individual and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Flour Cytometry. The following mouse monoclonal antibodies (mAbs) of IgG₁ subtype reacting with different integrin chains were used: 4B4 with the β_1 -chain

(Coulter, Hialeah, FL), 5E8 with the α_2 -chain, J143 with the α_3 -chain, Lv 230 with the α_v -chain, and MT78 with the α_6 -chain. MAb 439-9B recognizes the β_4 -chain.

ORS cells at $1 \times 10^6/\text{ml}$ were washed once with phosphate-buffered saline, 1% fetal calf serum, and 0.02% NaN_3 at 4°C and reconstituted with 1 ml of the same buffer. A 100 μl cell suspension was then incubated with 0.1 μg of mAbs or isotype control antibody (Dako, Glostrup, Denmark) for 25 min at 4°C . After being washed twice with the same buffer, cells were incubated with a phycoerythrin-labeled polyclonal goat anti-mouse anti-body (Dako) for another 25 min at 4°C , washed again, and subsequently fixed with the above-mentioned buffer supplemented with 2% paraformaldehyde. Cells were analyzed on a 4-logarithmic scale EPICS Profile II flow cytometer equipped with a power pack, and data were analyzed using the ELITE software (Coulter).

Epidermal Equivalents. ORS cells harvested from primary cultures were seeded at a density of $5 \times 10^5/\text{cm}^2$ on cell culture inserts (Falcon 3095) carrying 5×10^4 postmitotic fibroblasts on the undersurface of their microporous membrane. Culture medium was the same as for the preparation of primary cultures. After 24 hr, the ORS cells were exposed to air by aspiration of the medium inside the insert and then cultured for 12 to 14 days with three medium changes per week. In some cultures, 65 μM 5-bromo-2'-deoxyuridine (BrdU; Sigma) were added for the final 18 hr.

For histologic analysis, the epidermal equivalents were excised from the insert with a 6 mm punch (Stiefel Laboratorium, Offenbach am Main, Germany), fixed in 5% formalin, and processed further together with the underlying insert membrane according to standard procedures. For immunohistologic examination, the epidermal equivalents were similarly punched out, but then separated from the insert membrane by fine tweezers, snap-frozen in liquid nitrogen-cooled isopentane, and stored at -80°C until processing.

For indirect immunofluorescence, cryostat sections of 6 μm were air-dried, fixed with ice-cooled acetone/ethanol (1:1), rehydrated with phosphate-buffered saline, blocked for 15 min with nonimmune serum, and incubated at room temperature for 60 min with the primary antibodies and, after extensive washing, for

45 min with the secondary antibodies. The following mAbs were used as primary antibodies: Ks 8.60, mainly reacting with keratin (K) 10 and weakly with K1, diluted 1:20 (Sigma); anti-human involucrin, diluted 1:100 (Sigma); anti-human profilaggrin/filaggrin, diluted 1:100 (BTI, Stoughton, MA); 4B4 directed against the β_1 -integrin chain, diluted 1:10 (Coulter). Secondary mAbs against mouse IgG conjugated with fluorescein isothiocyanate were purchased from Sigma. As negative controls, sections were incubated with non-immune serum and conjugated secondary antibodies, which revealed in a few cases weak diffuse staining of fully keratinized areas.

For the determination of BrdU-positive cells, cryostat sections were denatured in 1.5 M HCl and successively incubated with 0.5 μ g/ml Hoechst 33258 for 30 min, mAb anti-BrdU (Partec, Arlesheim, Switzerland) diluted 1:100 for 45 min, and fluorescein isothiocyanate-linked anti-mouse IgG (Sigma) diluted 1:30 for 45 min. The percentage of BrdU-positive cells in the basal layer was determined in epidermal equivalents prepared from ORS cells of two leg ulcer patients aged 72 and 91 years ($n = 4$; two epidermal equivalents per patient). For each epidermal equivalent, about 2500 basally located nuclei in 10 randomly selected sections were counted.

For transplantation, the epidermal equivalents were excised from the insert together with the underlying membrane using a 6-mm punch (Stiefel Laboratorium) and positioned upside-down on a punched-out polyester membrane (Thomapor 95877; Reichelt Chemie, Heidelberg, Germany) of 6 mm diameter. In one patient, additional epidermal equivalents of 8 mm diameter were prepared likewise. The insert membrane together with the attached postmitotic fibroblasts was then carefully removed with fine tweezers. The epidermal equivalents on their supporting polyester membrane were washed in Dulbecco's phosphate-buffered saline and left floating therein until their application on the wound bed, usually for no longer than 30 min.

Autologous Grafting in Chronic Leg Ulcers. With the approval of the Ethics Committee of the University of Berne and after obtaining written informed consent, five in-patient (one male, four females, aged 58 to 91) suffering from recalcitrant chronic leg ulcers (four of them with more than two ulcers on the same leg, duration at least 4 years; venous or mixed arterial and venous disease in four. in one additional

diabetes mellitus, primary lymphoedema in one) were enrolled in a pilot study. The ulcers were cleaned conventionally (primarily with hydrocolloidal dressings and topical antimicrobial agents) until ready for grafting. Then up to 20 autologous epidermal equivalents, usually 6 mm, in one ulcer 8 mm in width, were placed, basal layer downward on the surface of the ulcers, and the supporting polyester membranes were carefully removed with fine tweezers. This grafting procedure was performed at the bedside; no anesthesia was needed. In four of the patients, further ulcers on the same leg served as controls. All ulcers were then covered with a transparent, semioclusive dressing (Tegaderm; 3M, London, Canada) overlaid by an elastic bandage with compression adapted to the patient's arterial status. The patients were immobilized for 2 h immediately after grafting. After 3 d, the semioclusive dressing was carefully removed and a hydropolymer dressing (Tielle: Johnson & Johnson Medical, Ascot, UK) applied, again overlaid by the elastic bandage. The hydropolymer dressings were then changed every 2 to 5 days. After complete re-epithelialization local treatment was switched to topical emollients, and the patients were instructed to adhere to a long-term compression therapy adapted to their arterial status. Take of the grafts and healing of the ulcers was documented by standardized photographs taken on each change of the dressings.

In Vitro ORS Cells Differentiate Into Epidermal Equivalents Similar to Normal Epidermis. Explanting plucked anagen hair follicles directly on the membrane of culture inserts carrying postmitotic fibroblasts as feeder cells at their undersurface proved to be a simple, efficient, and reproducible tool for establishing primary cultures of ORS cells. About 80% of the explanted hair follicles gave rise to outgrowth of ORS cells, even when derived from individuals aged up to 91 years. After 14 days, large areas of the insert were covered by compactly arranged small cells, at which time they were used for the preparation of the epidermal equivalents. In contrast, ORS cells derived from the trypsin-treated follicles exhibited a less compact arrangement with numerous cells of a larger size. Comparison of the growth behavior of 70 strains of ORS cells derived from 30 donors revealed no significant differences between young (21 donors aged 19 through 50 years) and old donors (9 donors aged 51 through 93 years), since about 0.5×10^6 cells were usually obtained

per explanted follicle. Cell viability was higher than 95%. In the absence of postmitotic fibroblasts, there were only sporadic outgrowth of ORS cells.

Because a logarithmic linear relationship between the relative level of β_1 -integrin on the cell surface and the proliferative capacity of keratinocytes has been postulated, we compared the expression of integrins in primary cultures of ORS cells established by the two different techniques, i.e., ORS cells from explanted follicles or from trypsin-treated follicles. ORS cells from four different donors grown by both techniques were analyzed by flow cytometry. On the basis of their light-scattering characteristics, the cells could be subdivided into two groups; group A, with a distinctly lower forward light scatter, i.e., smaller cell size, and group B, with higher forward light scatter, thus having a larger cell size. For ORS cells derived from explanted follicles, group A accounted for about 4% and group B for 72% of the total cell number, while values of 2.6% and 75%, respectively, were found for ORS cells grown from trypsin-treated follicles (mean values of four separate experiments). In group A, the percentage of cells staining for β_1 - β_4 -integrins as well as the mean fluorescence per cell of the β_1 - and to a lesser extent also the α_2 -, α_3 -, α_v -integrins, were higher in ORS cells grown from explanted follicles than in those from trypsin-treated follicles. In group B, no differences were detected in the two culture techniques, neither in the percentage of integrin-positive cells nor in the mean fluorescence per cell.

ORS cells harvested from primary cultures and plated on insert membranes carrying postmitotic fibroblasts at their undersurface developed a stratified epithelium within 14 days. This consisted of a basal layer of small cuboidal cells below a thick suprabasal compartment of progressively flattened cells. A granular layer and a mostly orthokeratotic horny layer were present.

The immunolocalization of epidermal differentiation products was identical to that found in normal epidermis. Thus, the differentiation-specific K10 was absent in the basal layer, but strongly expressed suprabasally from the second layer on. Involucrin displayed its typical honey-comb pattern from the mid-stratum spinosum on, whereas the granular staining of filaggrin formed a continuous band beneath the horny layer. As in normal epidermis, the reactivity of the α_2 -, α_3 - and β_1 -chains of

integrins was distributed over all aspects of the plasma membrane of the basal cells, displaying decreasing intensity with progressive differentiation.

BrdU-positive cells were found predominantly in the basal layer of the epidermal equivalents and accounted for 24% of the basal cells [597 ± 21 BrdU-positive cells for 2464 ± 115 basal cells (mean \pm SD): $n = 4$].

Based on 80% of follicles giving rise to ORS cell outgrowth, about five anagen hair follicles were needed to generate 1 cm^2 of epidermal equivalents. The period to generate graftable epidermal equivalents usually was 4 weeks *i.e.*, 2 weeks for the primary culture and 2 weeks for the organotypic culture.

Autologous Epidermal Equivalents Are Grafted Successfully on Chronic Leg Ulcers. A total of 11 ulcers were treated, seven of them by covering about 90% of the ulcer surface with densely arranged cultures, four by putting isolated cultures into the central parts. On the first change of the dressing 3 d after grafting, about 80% of the grafts were visible and adherent to the wound bed in both types of treatment. Within the following 2 to 3 wk the grafts consolidated in five of the seven densely grafted ulcers, resulting in complete re-epithelialization and healing. In the two remaining, chronically infected (*Pseudomonas*) ulcers, the grafts were partly destroyed, which led to delayed healing by 4 to 5 weeks. In the ulcers treated by isolated grafts, there was accelerated formation of granulation tissue and re-epithelialization mainly from the wound edges, as compared to the ulcers on the same leg treated with the dressings only. In this type of treatment, permanent take with subsequent expansion of the grafts resulting in complete re-epithelialization was only documented for one ulcer treated with larger epithelial sheets measuring 8 mm in diameter. The control ulcers in the four patients with more than two ulcers on the same leg were only slightly improved after 3 weeks, at which time they were treated either by further grafting of autologous epidermal equivalents or by conventional surgery.

After re-epithelialization, the epidermis was initially still fragile with some tendency to blistering after minor frictional trauma, occasionally resulting in small erosions. These erosions re-epithelialized rapidly under conventional topical treatment. The first patients have now been followed up for 6 mo and show increasing stabilization of the treated areas and no recurrence of the ulcers so far.

From the foregoing detailed description of the specific embodiments of the present invention, it should be readily apparent that a unique methodology for the selection and culture of keratinocytes from the outer root sheath (ORS) of hair follicles for subsequent use in, for example, skin grafting procedures, has been described. Although particular embodiments have been disclosed herein in detail, this has been done by way of example for purposes of illustration only, and is not intended to be limiting with respect to the scope of the appended claims which follow. In particular, it is contemplated by the inventor that various substitutions, alterations, and modifications may be made to the invention without departing from the spirit and scope of the invention as defined by the claims. For example, the selection of anagen hairs are believed to be a matter of routine for a person of ordinary skill in the art with knowledge of the embodiments described herein.

CLAIMS

1. A method for the treatment of a skin defect comprising
 - (a) culturing an intact hair follicle of an anagenic hair to obtain outer root sheath cells;
 - (b) culturing said outer root sheath cells to obtain keratinocyte precursor cells;
 - (c) preparing an epidermal or dermal equivalent comprising said keratinocyte precursor cells; and
 - (d) applying a portion of said epidermal or dermal equivalent to said defect.
2. The method of claim 1, wherein said outer root sheath cells are autologous cells obtained from an individual who will subsequently undergo treatment for a skin defect.
3. The method of claim 1, wherein said outer root sheath cells are homologous cells.
4. The method of claim 1, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing only homologous or autologous biological supplements.
5. The method of claim 1, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing human serum in a concentration of less than 5%.
6. The method of claim 1, wherein the culture density of said keratinocyte precursor cells is between about 3×10^4 cells/cm² and about 1×10^5 cells/cm².
7. The method of claim 1, wherein said epidermal or skin equivalents are coated on their top or cornified side with a fibrin glue.

8. The method of claim 1, wherein said epidermal or skin equivalents are coated on their top or cornified side with a carrier membrane.
9. The method of claim 1, wherein the graft thickness for said epidermal equivalents is 50-150 microns.
10. The method of claim 1, wherein the graft thickness for said complex skin equivalents does not exceed 0.4 mm.
11. A method for the treatment of a skin defect comprising
 - (a) culturing an intact hair follicle of an anagenic hair to obtain outer root sheath cells;
 - (b) culturing said outer root sheath cells to obtain keratinocyte precursor cells;
 - (c) preparing an epidermal or dermal equivalent comprising said keratinocyte precursor cells; and
 - (d) applying a portion of said epidermal or dermal equivalent to said defect wherein all culturing of cells is performed in a medium which utilizes autologous or homologous human serum in a concentration of preferably less than approximately 5%.
12. The method of claim 11, wherein said anagen or growing hair is cultured *in toto*.
13. The method of claim 11, wherein said keratinocyte precursor cells have a culture density of between approximately 3×10^4 cells/cm² to approximately 1×10^5 cells/cm².
14. The method of claim 11, wherein said outer root sheath cells are homologous cells.
15. The method of claim 11, wherein the culture density of said keratinocyte precursor cells is between about 3×10^4 cells/cm² and about 1×10^5 cells/cm².

16. The method of claim 11, wherein said epidermal or skin equivalents are coated on their top or cornified side with a fibrin glue.
17. The method of claim 11, wherein said epidermal or skin equivalents are coated on their top or cornified side with a carrier membrane.
18. The method of claim 11, wherein the graft thickness for said epidermal equivalents is 50-150 microns.
19. The method of claim 11, wherein the graft thickness for said complex skin equivalents does not exceed 0.4 mm.
20. A method for the treatment of a skin defect comprising
 - (a) culturing an intact hair follicle of an anagenic hair to obtain outer root sheath cells;
 - (b) culturing said outer root sheath cells to obtain keratinocyte precursor cells;
 - (c) preparing an epidermal or dermal equivalent comprising said keratinocyte precursor cells; and
 - (d) applying a portion of said epidermal or dermal equivalent to said defect wherein said epidermal or dermal equivalent is coated on its top or cornified side with a fibrin glue.
21. The method of claim 20, wherein said anagen or growing hair is cultured *in toto*.
22. The method of claim 20, wherein said outer root sheath cells are autologous cells obtained from an individual who will subsequently undergo treatment for a skin defect.
23. The method of claim 20, wherein said outer root sheath cells are homologous cells.

24. The method of claim 20, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing only homologous or autologous biological supplements.
25. The method of claim 20, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing human serum in a concentration of less than 5%.
26. The method of claim 20, wherein the culture density of said keratinocyte precursor cells is between about 3×10^4 cells/cm² and about 1×10^5 cells/cm².
27. The method of claim 20, wherein said epidermal or skin equivalents are coated on their top or cornified side with a fibrin glue which contains one or more anti-microbial, anti-fungal, or anti-viral agents emulsified therein.
28. A method for the selection of keratinocyte precursor cells from the outer root sheath of hair for subsequent use in a composition for healing a skin defect, comprising the steps of:
 - (a) plucking of an intact anagen hair;
 - (b) primary-culturing the outer root sheath-derived keratinocyte precursor cells by adhering said intact anagen hair to a microporous membrane, which possesses growth-arrested/limited feeder cells on its undersurface so as to select for keratinocyte precursor cells from the outer root sheath of hair;
 - (c) organotypically-culturing the outer root sheath cells harvested from said primary cultures by inoculating a microporous membrane which also possesses growth-arrested/limited feeder cells on its undersurface;
 - (d) generating an epidermal or complex skin equivalent, for subsequent use as a graft insert, by placing a carrier membrane on top of said organotypic-culture from step (c) and detaching said complex skin or epidermal equivalent, which is comprised

of the keratinocyte precursor cells and carrier membrane, together as a single, laminar unit;

- (e) contacting said epidermal or skin equivalent with a skin defect present on an individual, and immobilizing said epidermal or skin equivalent at the site of contact.

29. The method of claim 28, wherein said outer root sheath cells are autologous cells derived from the individual who will subsequently undergo treatment for a skin defect.
30. The method of claim 28, wherein said outer root sheath cells are homologous cells.
31. The method of claim 28, wherein the culture density of said keratinocyte precursor cells is between about 3×10^4 cells/cm² and about 1×10^5 cells/cm².
32. The method of claim 28, wherein the culture density of said growth-arrested/limited feeder cells on said microporous membrane is between about 1×10^4 cells/cm² and about 5×10^4 cells/cm².
33. The method of claim 28, wherein said growth-arrested/limited feeder cells are banked or immortalized cells.
34. The method of claim 28, wherein said primary and organotypic cultures utilize autologous or homologous human serum.
35. The method of claims 28, wherein said primary and organotypic cultures utilize autologous or homologous human serum in a concentration of less than about 5%.
36. The method of claim 28, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing only homologous or autologous biological supplements.

37. The method of claim 28, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing only homologous or autologous releasates from blood components.
38. The method of claims 28, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing only homologous or autologous releasates from blood components at a concentration of about 0.1% to about 20%.
39. The method of claim 28, wherein said epidermal equivalents are coated on their top or cornified side with a fibrin glue.
40. The method of claims 28, wherein said epidermal equivalents are coated on their top or cornified side with a carrier membrane.
41. The method of claims 28, wherein the graft thickness for said epidermal equivalents is 50-150 microns.
42. The method of claims 28, wherein the graft thickness for said complex skin equivalents does not exceed 0.4 mm.
43. The method of claim 28, wherein said microporous membrane is coated by one or more extracellular matrix substances selected from a group consisting of: fibrin, fibronectin, collagens, laminins and hyaluronan.
44. The method of claims 28, wherein said microporous membrane possesses a growth-arrested/limited feeder cell system on its undersurface with said feeder cells of at least one type of cell selected from the group comprising human dermal fibroblasts, epidermal cells, mesenchymal cells, neuronal cells and endothelial cells.

45. The method of claim 28, wherein said carrier membrane is made from one or more types of materials selected from the group comprising polyester, PTFE, polyurethane, hyaluronic acid, polylactic acid, collagen, or a silicone or vaseline gauze dressing.
46. The method of claim 28, wherein the size of said epidermal equivalent is selected from the group consisting of 1.0 cm, 1.5 cm, 2.0 cm, and 2.5 cm in diameter.
47. A method of shipping or transporting epidermal equivalents comprising:
 - (a) detaching said epidermal equivalents from a culture medium, and
 - (b) transferring said epidermal equivalents onto a transport medium.
48. The method of claim 47, wherein said epidermal equivalents are coated on their top or cornified side with a carrier membrane.
49. The method of claim 48, wherein said epidermal equivalents are further sealed and shipped for future use in grafting.
50. The method of claim 47, wherein said transport medium comprises a solidified or gelled medium.
51. The method of claim 50, wherein said solidified or gelled medium is selected from the group consisting of agarose, methyl cellulose, or another gelifying substance.

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(54) Title: IMPROVED KERATINOCYTE CULTURE AND USES THEREOF

(57) Abstract: The present invention relates to the treatment of skin defects by organotypically-cultured autologous keratinocytes isolated from the outer root sheath of anagen or growing hair. Methods for primary, as well as subsequent organotypic cultures (i.e., epidermal equivalents) in fully-defined media supplemented by autologous human serum and substances isolated from blood components, with minimal allogeneic biological supplements, are disclosed herein. Techniques to prepare epidermal equivalents for transplantation by use of a biocompatible glue are also disclosed herein.

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#3

IN THE UNITED STATES PATENT AND TRADEMARK OFFICEAPPLICANTS: Hunziker *et al.*

FOR: IMPROVED KERATINOCYTE CULTURES AND USES THEREOF

Assistant Commissioner for Patents
Washington, D.C. 20231**COMBINED DECLARATION AND POWER OF ATTORNEY**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and sole inventor of the subject matter (an original, first and joint inventor) which is claimed and for which a utility patent is sought on the invention entitled:

IMPROVED KERATINOCYTE CULTURE AND USES THEREOF

the specification of which was filed on January 18, 2002, and assigned Serial No. 10/031,183.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

- ☒ I hereby claim the benefit under Title 35, United States Code, § 119(e) or §120 of any United States applications listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

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John M. Garvey	<u>37,833</u>	Brian Rosenbloom	41,276
James G. Gatto	<u>32,694</u>	Robert J. Sayre	42,124
Richard Gervase	<u>46,725</u>	C. Eric Schulman	43,350
Matthew J. Golden	<u>35,161</u>	Gregory J. Sieczkiewicz	48,223
Sonia K. Guterman	<u>44,729</u>	Thomas M. Sullivan	39,392
John A. Harre	<u>37,345</u>	Janine Susan	46,119
Brian P. Hopkins	<u>42,669</u>	Nicholas P. Triano III	36,397
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issued thereon.

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